{Exhibit 68}

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ANALYTICAL BIOCHEMISTRY 16, 481–486 (1966)

A New Fluorescence-Yielding Substrate for Alkaline and Acid Phosphatase

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In the search for an ultrasensitive method for the determination of alkaline phosphatase to be incorporated in an automated system, the ammonium salt of flavone 3-diphosphate was synthesized and found to be a stable, versatile, and sensitive substrate for assaying both acid and alkaline phosphatase.

Flavone 3-monophosphate was also synthesized, and exhibited comparable sensitivity to the diphosphate. The enzymic cleavage of the mono- and diphosphate groups liberates 3-hydroxyflavone, which can be assayed by direct absorption at 410 mμ or, with greater sensitivity, fluorometrically at 510 mμ. Ultrahigh sensitivity can be achieved by forming the fluorescent metallic chelate of 3-hydroxyflavone with an excess of aluminum ions at a slightly acidic pH value. After an incubation period of 5 min, approximately 10^{-11} gm of phosphatase can be assayed by the chelate method. The substrate appears to have excellent cytohistochemical fluorescent stain applications. The fluorogenic phosphatase substrate 3-*O*-methylfluorescein phosphate (1), although more sensitive, lacks the desired stability in solution for automation. The substrate, β-naphthyl phosphate (2–4), has been investigated and was found in our laboratory to be less sensitive than flavone 3-diphosphate.

METHOD AND MATERIAL

Diphosphate Synthesis

A 1.2 gm sample of reagent-grade 3-hydroxyflavone was dissolved in 15 ml of dry tetrahydrofuran and added slowly at 20°C to 0.7 ml of phosphorus oxychloride in 50 ml of dried benzene. Approximately 2.5 ml of triethylamine (TEA) was added to the reaction mixture, i.e., a sufficient amount of TEA to give an apparent pH of 6. After a reaction time of 5–6 min, 50 ml of water was added to the mixture and the alkalinity raised to pH 8 with 5% NH_4OH. The solution was treated twice with

75 ml of a mixture containing four parts hexane to one part 1-butanol. The organic phase was discarded and the aqueous phase lyophilized. The dried material was redissolved in 20 ml of water and precipitated twice at pH 8 by addition of 1 N NH_4OH with an excess of isopropyl alcohol. The yellow contamination remained in solution while the white powder precipitate was filtered through a Büchner funnel with Whatman No. 40 filter paper, vacuum-dried, and stored in a screw-cap vial.

Monophosphate Synthesis

In the preparation of dibenzyl chlorophosphonate (5), exactly 1.30 gm of dibenzyl phosphite was added to 0.67 gm of *N*-chlorosuccinimide in 15 ml of dry benzene with stirring for 10 min. The reaction mixture was allowed to stand at room temperature for 2 hr. The succinimide was filtered and the freshly prepared dibenzyl chlorophosphonate kept free from moisture. The phosphonate was added to 1.2 gm of 3-hydroxyflavone in 50 ml of dry benzene. A slight molar excess of triethylamine was added to the reaction mixture at 60°C and run for 2 hr. Triethylamine hydrochloride was removed by filtering the warm solution. The filtrate was evaporated to near dryness and extracted with 50 ml of ethanol. The ethanolic solution was hydrogenated with 50% platinum-paladium black at 50° for 2–3 hr. The monophosphate was filtered, dried, redissolved in hot water, and filtered while hot. Upon cooling, the solution was extracted twice with benzene and a 1,4-butanol-hexane mixture. After extraction, the aqueous phase was lyophilized and redissolved in 10 ml of distilled water. A 0.45 μ Millipore filter was used to separate the insoluble yellow impurity. The product, monophosphate, was lyophilized to a dry powder.

Colorimetric Assay

Alkaline phosphatase activity was determined with the flavone 3-diphosphate ammonium salt photometrically by reading the change in optical density of a 0.005 mg/ml substrate solution after an incubation period of 5 min. The concentrations of *Escherichia coli* alkaline phosphatase (Sigma Chemical Co.) ranged from 0.03 to 0.5 μg/ml. The reaction was carried out in 2.0 ml of a 0.05 M borate buffer solution at pH 9.8, which is optimum for enzyme activity. A Beckman DB spectrophotometer set at 410 mμ was used to monitor the reaction.

Direct Fluorescence of Cleaved Substrate

Borate buffer quenched the fluorescence of the liberated 3-hydroxyflavone; consequently a 0.025 M glycine buffer of pH 9.8 was used for the direct fluorometric and the chelate procedures. The direct fluorescent assay of phosphatase was carried out by the addition of 20 μl of a 1 mg/

ml substrate solution to 2 ml of glycine buffer and varying the amount of enzyme. The reaction was monitored by an Aminco-Bowman spectrophotofluorometer activated at a wavelength of 360 mμ with emission at 510 mμ. If necessary, the final fluorescence of the reaction can be increased 200-fold by addition of an equal volume of ethanol.

Fluorescent Chelate Procedure

Trace amounts of purified alkaline phosphatase were determined fluorometrically by formation of the fluorescent aluminum chelate from the cleaved substrate. The basic procedure was to add 20 μl of the 1 mg/ml diphosphate solution in a cuvet containing 0.5 ml of 0.025 *M* glycine buffer. Amounts of 10^{-12} to 10^{-7} gm of *E. coli* alkaline phosphatase were added to the buffered substrate solution. After the prescribed reaction time of 5 min, the enzyme reaction mixture was treated with 2 ml of a mixture of 3 parts 0.05 *M* acetic acid buffer, pH 5.0, and 2 parts 0.01 *M* aluminum chloride. Two minutes was allowed for optimum chelate development and, with activation at 400 mμ, maximum fluorescent emission occurred at 450 mμ.

In the determination of acid phosphatase (Mann Research Laboratory) by the chelate method, the reaction was carried out at pH 5.0 in the presence of aluminum. The increase in fluorescence could be followed directly as the enzymic reaction proceeded.

Cytohistochemical Application

Buccal epithelial smears, and *Serratia marcescens, Bacillus subtilis* and *Rhizopus nigricans* culture smears were prepared on microscope slides. After drying, the smears were bathed for 5 min with a 10 mg/ml flavone 3-diphosphate ammonium salt solution in either water or pH 9.8 borate buffer. The slides were observed under a Reichert fluorescent microscope using a KG 2, BG 12, and UG excitation filter combination and a GG 9 Schott glass emission filter.

RESULTS AND DISCUSSION

The ammonium salt of flavone 3-diphosphate was obtained in high yields of above 90% of theoretical. When dried, it is a white powder having a melting point of 170–172°C with decomposition at 160°. A microanalysis of the compound was consistent with the diphosphate structure, except for the possibility of contamination by equal amounts of the mono- and triphosphates. High purification of the compound was difficult to obtain by conventional methods. *Found:* C 39.1, H 5.4, P 13.0, N 9.1; *theoretical:* C 40.1, H 4.7, P 13.8, N 9.4. The flavone 3-monophosphate was obtained in yields of only 10% of theoretical. *Found:* C 57.1, H 3.6,

P 9.7; *theoretical:* C 56.6, H 3.8, P 9.7. Therefore, the diphosphate was the compound of choice because of the ease of synthesis, high yields, and comparable sensitivity. The ultraviolet spectra of the mono- and diphosphate compounds were identical. Strong absorption occurred in the infrared at 970 and 1100 cm^{-1} for the diphosphate, which indicates a pyrophosphate linkage and multiple phosphate bonds.

Alkaline phosphatase as well as acid phosphatase reacted as rapidly with the flavone 3-diphosphate as the disodium phenyl phosphate, thymolphthalein phosphate, or β-naphthyl phosphate. A Michaelis constant of 8.8×10^{-6} was determined for the diphosphate while that obtained from β-naphthyl phosphate was 8.1×10^{-6}.

With the optical absorption technique, as little as 0.03 μg of enzyme could be detected within 5 min (Fig. 1), whereas with the direct fluorometric method the sensitivity was slightly better (Fig. 2). However, concentrations of 10^{-11} gm of *E. coli* alkaline phosphatase could be detected within 5 min using the aluminum chelate method (Fig. 3). The enzyme reaction proceeds at a constant rate in the presence of excess substrate. The 5 min period is an arbitrary incubation time chosen for convenience. In the chelate method, a large excess of aluminum to sub-

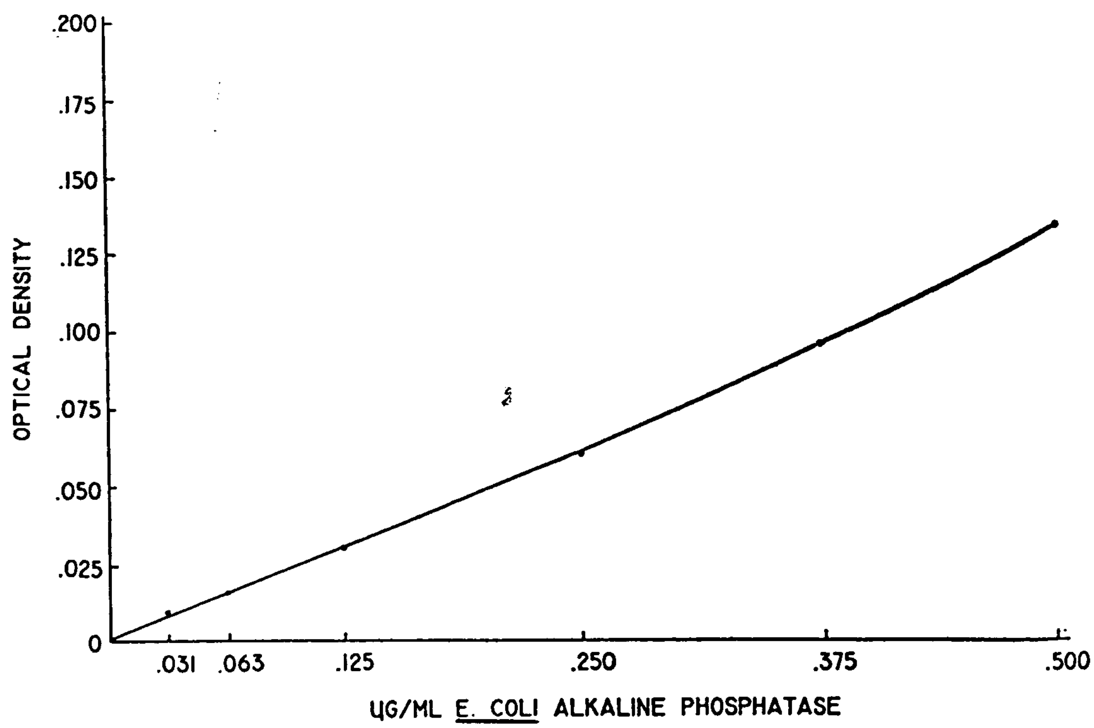

FIG. 1. Colorimetric method for alkaline phosphatase. Plot of absorption versus enzyme concentration in 2 ml of borate buffer incubated at room temperature for 5 min. Optical density was recorded at 410 mμ.

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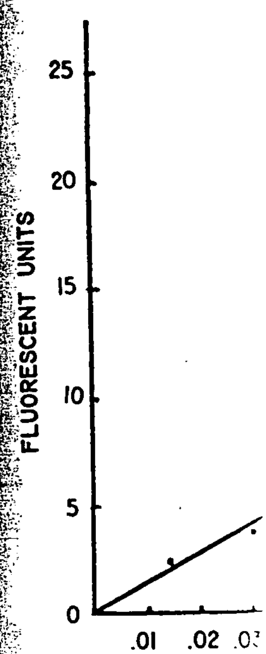

FIG. 2. Plot of
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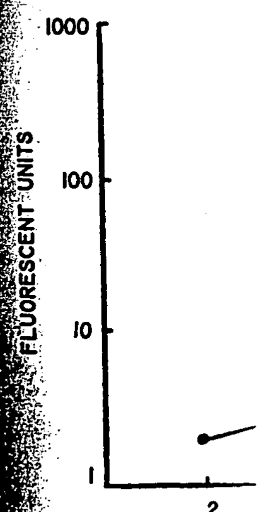

FIG. 3. Log-log
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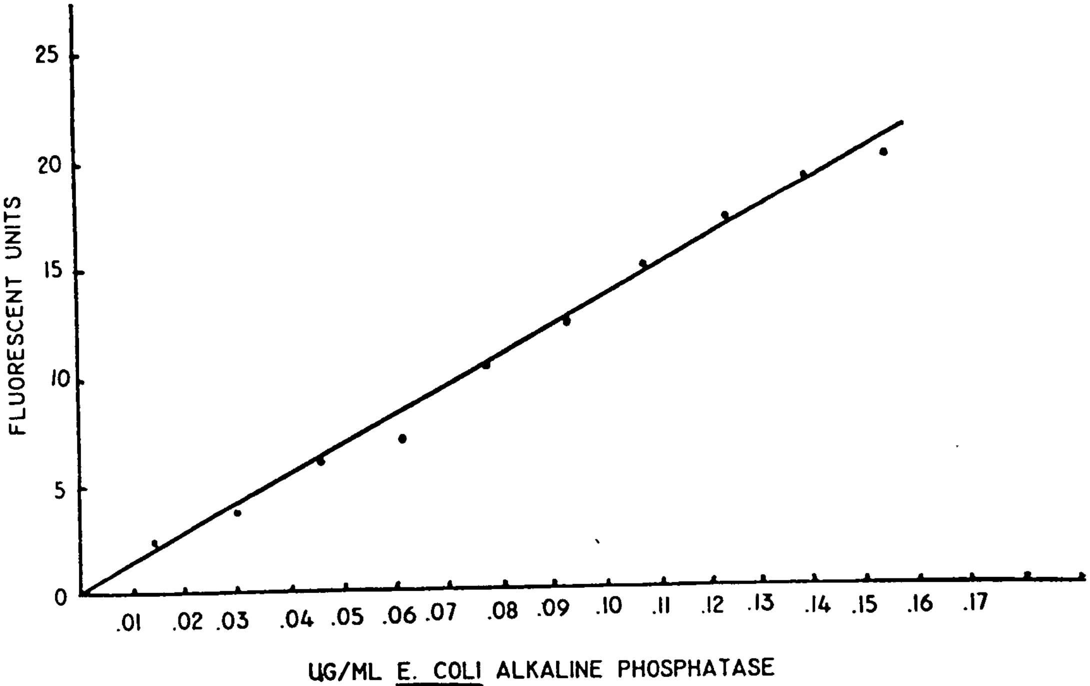

FIG. 2. Plot of direct fluorescence of enzyme-liberated 3-hydroxyflavone. The reaction was carried out at room temperature in borate buffer. Fluorescence was recorded after 5 min at 510 mμ with an activation wavelength of 360 mμ.

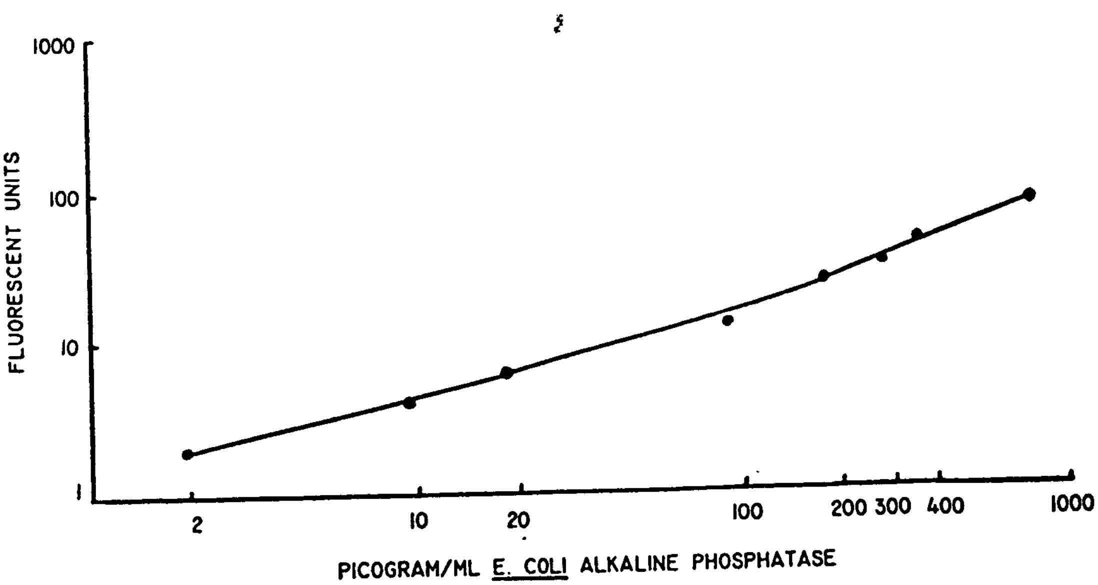

FIG. 3. Log-log plot of fluorescent chelate assay for alkaline phosphatase. Various enzyme concentrations were added to 0.5 ml of glycine buffered substrate with a 5 min incubation period; 2 ml of aluminum chloride solution was added and the fluorescent intensity recorded in 2 min. Fluorescence was measured at 450 mμ with activation at 400 mμ.

strate is required for maximum fluorescence and to prevent competition from the liberated phosphate for the aluminum of the chelate. At low concentrations, the aluminum reacts with the ligand in a 1:3 molar ratio; however, at higher aluminum concentrations as in the phosphatase assay the system becomes complex, as found with beryllium-morin (6). The fluorescence of the water-soluble chelate is linear with concentration in dilute solution.

In the direct method, fluorescence measured at pH 9.8 is linear for dilute concentrations of free 3-hydroxyflavone up to 3 μg/ml. At pH 5.0, the fluorescence of the cleaved substrate is 8 times greater than at pH 9.8, but at the low pH fluorescence is not linear with concentration. The fluorescence and optical density of 3-hydroxyflavone are not linear at high concentrations due to the insolubility of the compound in aqueous solution.

In comparing the stability of the fluorescein and flavone derivatives, a 1.0 mg/ml pH 9.8 solution of flavone diphosphate remains stable at 23°C for a minimum of 72 hr with no significant decomposition. At room temperature, the background fluorescence of a 0.1 mg/ml solution of 3-*O*-methylfluorescein at pH 8.0 increases 7-fold in 24 hr due to spontaneous hydrolysis.

Cytochemical observations with the diphosphate demonstrated that the substrate is active in both mammalian and bacterial cells. The cells fluoresce brightly with relatively low background. The specificity of the substrate for phosphatase has not yet been determined.

It is suggested that other substrates capable of chelation upon enzymic cleavage be investigated to enhance sensitivity.

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REFERENCES

1. Tierney, J. H., *Federation Proc.* **23,** 2381 (1964).
2. Greenberg, L. J., *Biochem. Biophys. Res. Commun.* **9,** No. 5, 440 (1962).
3. Moss, D. W., *Clin. Chim. Acta* **5,** 283 (1960).
4. Guilbault, G. G., and Kramer, D. N., *Anal. Chem.* **37,** No. 13, 1675 (1965).
5. Atherton, F. R., Howard, H. T., and Todd, A. R., *J. Chem. Soc.* 1106 (1948).
6. Fletcher, M. H., *Anal. Chem.* **37,** No. 4, 550 (1965).